Clinical Correlates of Circulating Immune Complexes and Antibody Reactivity in Squamous Cell Carcinoma of the Head and Neck

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Purpose: To evaluate the correlation between the presence and titer of host-derived antibody reactivity, circulating immune complexes, and clinical course and prognosis in patients with squamous cell carcinoma of the head and neck (SCCHN).

Materials and Methods: Serum samples, obtained from untreated patients with squamous cell carcinoma of the larynx entered onto a multiinstitutional trial, were evaluated for the presence of elevated circulating immune complexes (221 patients) and host-derived antibody directed against two SCCHN cell lines (107 patients).

Results: Patients had significantly elevated levels of circulating immune complexes as measured by C1q binding compared with normal controls. Patients with higher levels of circulating immune complexes were less likely to respond to chemotherapy. No correlations were noted between immune complex levels and stage of disease, nodal status, site of disease, recurrence, or survival. Evaluation of native antibody titers for their relationship to clinical correlates showed no statistically significant associations. In sera subjected to immune complex dissociation, patients with moderately or poorly differentiated tumors had significantly higher antibody titers when compared with patients with well-differentiated tumors. Because marked variation in the increase of antibody titers following immune complex dissociation was noted, the ratio of immune complex–dissociated to native antibody titer was examined. Patients with a high ratio had a lower proportion of complete and partial responses to chemotherapy.

Conclusion: Our results support the conclusion that the formation of tumor-associated immune complexes in patients with SCCHN is associated with a decreased response to chemotherapy.

J Clin Oncol 11:2427-2433.

AN UNDERLYING, but still unproven, assumption of tumor immunology is that antigens present on cancer cells are quantitatively or qualitatively different from those on normal cells. The definition and characterization of these tumor-associated antigens have been a major focus of investigations for more than two decades. The hope is that determination of the nature and significance of these antigens may allow the development of more effective diagnostic and therapeutic agents.

Serologic studies in patients with squamous cell carcinoma of the head and neck (SCCHN) have been limited. While some investigators have documented changes in systemic immunoglobulin levels in patients with late-stage disease, the lack of reproducible results has made these observations difficult to interpret.[1,2] In studies performed in our laboratories, autologous antibody reactivity against SCCHN has been noted in 24 of 41 systems tested with a median titer of 1:4.[3,4] In the majority of cases, autologous antibody reactivity could only be detected in undiluted serum, precluding further analysis. The low incidence and weak titer of autologous antibody to SCCHN have raised questions regarding the relevance of humoral immunity. The presence of circulating antigen in patients and the resulting formation of immune complexes may explain these difficulties in detecting autologous antibody.

Circulating immune complex levels were measured in patients with SCCHN.[5-10] As with other tumors, elevated levels of immune complexes were noted and, in some instances, correlated with prognosis and clinical course. Schantz et al[11] evaluated 43 previously untreated patients with head and neck cancer. It was noted that significantly higher levels of circulating immune complexes, as measured by C1q binding, were noted in patients with SCCHN who did not respond to chemotherapy. This observation has been confirmed by others[12] using polyethylene glycol precipitation to measure circulating immune complexes.

We have turned to circulating immune complexes in the sera of cancer patients as a new source of host-derived serologic reagents. We have demonstrated that dissociation

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Submitted March 9, 1993; accepted August 24, 1993.

Supported in part by National Cancer Institute, Bethesda, MD, grant no. RO1 CA49708, the Cooperative Studies Program of the Veterans Affairs Medical Research Service, and Bristol-Myers Squibb Co, New York, NY.

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0732-183X/93/1112-0019$3.00/0

of immune complexes by acidification and ultrafiltration (AD&U) of sera augments autologous antibody reactivity in the majority of cases studied.[3,13] Of the 21 melanoma and eight SCCHN systems studied, augmented autologous antibody titers directed against the patient's own tumor were found following AD&U in all but one case. It would appear that autologous antibody directed against SCCHN and melanoma is the rule rather than the exception, and may be indicative of a more generalized phenomenon. These findings support the hypothesis that host-derived antibody to SCCHN and melanoma may be obscured by circulating antigen and that immune complexes may provide a readily accessible source of relevant antigen and antibody.

In melanoma, we have noted correlations between the presence and titer of host-derived antibody reactivity and clinical course and prognosis.[14,15] Serial studies have also been performed on six patients with SCCHN and, as with melanoma, correlations with clinical course were noted.[16] To delineate better the role circulating immune complexes and host-derived antibody and antigen play in SCCHN, we have sought to study a larger, well-defined cohort of patients. We now report our serologic evaluation of patients with untreated stage III and IV squamous cell carcinoma of the larynx prospectively studied as part of a controlled, clinical trial.

MATERIALS AND METHODS

Patient Population

Three hundred thirty-two patients with stage III and IV squamous cell carcinoma of the larynx were entered into a prospective, multiinstitutional randomized trial sponsored by the Cooperative Studies Program of the Department of Veterans Affairs. The results of this trial have been reported previously.[17] There were no significant differences between treatment groups on baseline demographics and tumor characteristics. Briefly, patients were randomized to receive either three cycles of chemotherapy (cisplatin and fluorouracil) and radiation therapy, or surgery and radiation therapy. Patients were assessed for response, toxicity, and overall survival. No statistically significant differences in disease-free and overall survival were observed. The pattern of initial tumor recurrence differed, with more local recurrences and fewer distant metastases noted in patients who received chemotherapy.

Serologic Methods

Serum samples were obtained at study entry before therapy. Serum was obtained from clotted blood, aliquoted, and stored at −70°C. Serum samples were available for circulating C1q binding from 221 patients and immune complex dissociation from 107 patients of the 332 patients entered onto the protocol. One hundred four of 107 samples used to measure antibody titers were the same as those used to measure C1q binding.

SCCHN cell lines. The method reported by Krause et al[18] was developed in our laboratory for establishing SCCHN cell lines. Squamous cell carcinoma lines UM-SCC-23 and UM-SCC-17, known to express the 60-kd SCCHN-associated antigen detected by autologous antibody, were used.[19]

Protein A hemadsorption. The protein A hemadsorption assay was performed according to the method reported by Pfreundschuh et al.[20] Briefly, indicator cells for the protein A–mixed hemadsorption tests were prepared by conjugating staphylococcal protein A (Pharmacia Fine Chemicals, Piscataway, NJ) to the surface of selected human blood group O-Rh–positive RBCs with 0.01% $CrCl_3$ at pH 5.0. Indicator cells were washed two times in phosphate-buffered saline (PBS) plus 1% γ-globulin–free fetal calf serum (FCS) (GIBCO, Grand Island, NY) and resuspended for use in this medium. Target monolayers were seeded in micro-Terasaki assay plates at a concentration of 300 to 500 cells per well and allowed to adhere for 24 hours. After incubation of target cells with 0.01 mL of sera at 37°C, wells were washed three times with PBS containing 2% γ-globulin–free FCS at 37°C and 0.01 mL of an 0.15% suspension of indicator cells was added to each well. Plates were washed two to four times with PBS plus 2% γ-globulin–free FCS after 45 minutes; positive cells were those with a greater than 50% erythrocyte rosette. The end point of the assay was the last well with 10% of target cells (positive). Because maximum antibody titers reflect doubling dilutions, a wide standard deviation was noted.

AD&U. The method initially reported by Sjogren et al[21] was used to dissociate immune complexes. To assure uniform processing of sera, an eight-chamber multichannel ultrafiltration apparatus (Amicon Corp, Danvers, MA) was used. Each chamber was fitted with an HP-30 membrane (Amicon Corp), with a molecular weight exclusion of 100,000. One milliliter of serum was added to each chamber and AD&U was performed with glycine-saline buffer (0.1 mol/L, pH 3.1) at 4°C under 30 lb/in^2 N_2. AD&U was continued until 10 times the serum volume was reached. Studies have shown that ultrafiltration of that volume allows maximal augmentation of antibody reactivity following removal of the circulating SCCHN-associated antigen.[22] The serum was then washed with the same volume of PBS to correct pH. Maximum antibody titers were determined by performing quadruplicate assays against each cell line (a total of eight assays for each serum sample). The incidence and titer of antibody to SCCHN were compared in relation to a number of prognostic variables. Previously, we have reported that sera from six normal individuals did not react with SCCHN cell lines both before and after immune-complex dissociation.[3]

C1q-binding test. Measurement of immune complexes used C1q binding as previously described.[11] C1q, purified by the method reported by Kolb et al,[23] was radiolabeled with iodine 125 by the iodobead method (Pierce Chemical Co, Rockford, IL). The C1q-binding test was performed as previously described, using edathamil-treated sera according to the method reported by Zubler et al.[24] Results were expressed as micrograms per milliliter of purified immunoglobulin G, aggregated at 63°C for 30 minutes at a concentration of 3 mg/mL and diluted serially in heat-inactivated (56°C for 30 minutes) normal donor serum. Sera from healthy, age-matched donors were used as negative controls and sera from patients with rheumatoid arthritis provided positive controls in each test run.[25]

Patient Characteristics

Patient characteristics of the parent protocol have been published previously.[17] Briefly, of the 332 patients entered, 50% were randomized to the surgery arm. There were 188 stage III and 144 stage IV patients. When grouped by nodal involvement, 180 were N0, 60 N1, 37 N2, and 55 N3. Supraglottic lesions accounted for 208 of the

Table 1. Patient Characteristics

Clinical Characteristics	Clinical Trial Patients (%)		C1q Patients		Antibody Titer Patients	
	No.	%	No.	%	No.	%
Stage						
III	188	57	131	59	63	59
IV	144	43	90	41	44	41
Nodal status						
N0-N1	240	72	163	74	81	76
N2-N3	92	28	58	26	26	24
Site						
Glottic	124	37	81	37	43	40
Other	208	63	140	63	64	60
Differentiation						
Well	31	12	26	14	16	15
Moderate/poor	233	88	159	86	81	85
Treatment						
Surgery	166	50	115	52	49	46
Chemotherapy	166	50	106	48	58	54

patients entered onto the parent trial. Evaluation of the 221 and 107 patients whose sera were used to measure C1q binding and antibody reactivity, respectively, showed no statistically different characteristics when compared with patients entered onto the parent clinical trial. The sole criterion for patient serum selection into this laboratory-based study was the availability of sufficient serum for analysis. A summary of patient characteristics is listed in Table 1.

Serologic Studies

Serum samples were tested for C1q binding and antibody titers in native and immune complex–dissociated sera. Results were scored by investigators blinded to the patient's clinical status.

Statistical Analysis

Data were analyzed using parametric techniques. Differences in continuous titer levels between dichotomous, independent groups were detected using Student's *t* test. The χ^2 test was used to examine differences in grouped titer levels (high *v* low) by disease characteristics. Disease-free and overall survival of patients by grouped titer levels were examined by Kaplan-Meier techniques; the log-rank test was used to detect differences between survival curves. A two-sided alpha level of .05 was considered statistically significant for all analyses.

RESULTS

Circulating Immune Complex Levels

Circulating immune complex levels were evaluated in 221 patients entered onto the parent protocol. Patients with SCCHN were noted to have significantly elevated levels of circulating immune complexes as measured by C1q binding as compared with normal controls (94.10 ± 114 *v* 31.46 ± 43 μg/mL, $P = .0001$) (Table 2). No correlations were noted between immune complex levels and either stage of disease, nodal status, site of disease, recurrence, or survival. Patients who did not respond to chemotherapy had higher levels of circulating immune complexes than nonresponders; however, the difference was not significantly significant ($P = .338$). However, as listed in Table 3, patients whose C1q-binding levels were greater than 115 μg/mL (mean ± 2 SD of normal controls) were less likely to respond to chemotherapy ($P = .049$), which is consistent with our previously reported results.[11] No significant differences in tumor stage, nodal stage, tumor differentiation, etc were noted between the two groups.

Table 2. Circulating Immune Complex Levels: Clinical Correlates

Variable (n)	Mean C1q-Binding Level μg/mL	P
Overall		
Patients (221)	94.10 ± 114	
Controls (132)	31.46 ± 43	.0001
Stage		
III (131)	97.60 ± 104	
IV (90)	91.69 ± 120	.706
Nodal status		
N0-N1 (163)	94.89 ± 115	
N2-N3 (58)	91.86 ± 111	.862
Site		
Glottic (81)	111.46 ± 134	
Supra/subglottic (140)	84.05 ± 99	.112

Antibody Titers

The maximum antibody titer of native sera (T1) and sera subjected to AD&U (T2) from 63 stage III and 44 stage IV patients were tested against allogeneic cultured SCCHN cell lines UM-SCC-17 and UM-SCC-23. Both of these cell lines had previously been shown to express a 60-kd SCCHN-associated antigen recognized by patients with SCCHN.[19] In no case were discrepancies in antibody titers of more than one doubling dilution in the determined maximal antibody titer observed.

In native serum, mean maximum antibody titers were noted to be 333 ± 418. Titer levels were examined by stage of disease, nodal class, site of primary tumor, and histology (Table 4). Patients were monitored for response to chemotherapy, recurrence, disease-free survival, and overall survival. When T1 titers were evaluated for their

Table 3. Association Between Circulating Immune Complex Levels and Response to Chemotherapy

Response	C1q Level μg/mL ≤ 115 No.	≤ 115 %	> 115 No.	> 115 %	P
CR	29	20	13	4	
PR	59	40	59	19	
NR	12	8	28	9	.049

Abbreviations: CR, complete response; PR, partial response; NR, no response.

Table 4. Host-Derived Mean Antibody Titers

Variable (n)	T1 (1/N) Mean ± SD Titer	P	T2 (1/N) Mean ± SD Titer	P	T2/T1 Mean ± SD Ratio	P
Stage						
III (63)	298 ± 418		1,604 ± 1,451		310 ± 923	
IV (44)	381 ± 420	.316	2,145 ± 1,973	.129	144 ± 460	.228
Nodal status						
N0-N1 (81)	352 ± 488		1,632 ± 1,437		283 ± 849	
N2-N3 (26)	307 ± 312	.569	2,073 ± 1,970	.205	113 ± 416	.181
Site						
Glottic (43)	296 ± 477		1,520 ± 1,311		284 ± 771	
Other (64)	357 ± 377	.486	2,031 ± 1,895	.104	214 ± 715	.644
Differentiation						
Well (16)	254 ± 272		756 ± 492		24 ± 64	
Moderate/poor (81)	360 ± 448	.215	2,045 ± 1,794	.0001	244 ± 757	.011
Recurrence, surgery patients						
No (37)	332 ± 408		1,810 ± 1,675		322 ± 957	
Yes (12)	353 ± 282	.848	1,234 ± 1,452	.264	6 ± 9	.052
Recurrence, chemotherapy patients						
No (30)	261 ± 289		1,915 ± 1,479		401 ± 944	
Yes (27)	406 ± 586	.251	2,002 ± 2,045	.856	62 ± 199	.064
Recurrence, all patients†						
No (67)	297 ± 358		1,845 ± 1,570		357 ± 944	
Yes (39)	389 ± 509	.321	1,766 ± 1,897	.818	45 ± 167	.010

NOTE. T1, maximum antibody titers in native sera; T2, maximum antibody titers immune complex–dissociated sera.

*Ten patients not evaluated for histology and removed from analysis.

†One patient not assessable for recurrence.

relationship to disease characteristics, no statistically significant associations were noted. A similar analysis was performed with immune complex–dissociated sera. Mean T2 antibody titers were 1,824 ± 1,695. Consistent with previous studies, antibody titers in sera subjected to AD&U were higher than native serum titers.[3,22] No statistically significant associations with tumor characteristics or outcome were noted with the exception of histology. Patients with moderately or poorly differentiated tumors had significantly higher T2 antibody titers when compared with patients with well-differentiated tumors (2,045 ± 1,794 *v* 756 ± 492, $P = .0001$).

Ratio of Native to Immune Complex–Dissociated Serum Antibody Titers

We noted that there was marked variation in the relative increase of T2 titers following AD&U. As the T1 and T2 determination were performed on the same serum samples, a marked increase in T2 titers relative to T1 titers would reflect a greater degree of antibody bound to circulating antigen. For example, a patient who had a T1 titer of 1:1,000 and a T2 titer of 1:2,000 would not have as high a T2-to-T1 ratio as a patient whose T1 and T2 titers were 1:4 and 1:500, respectively. To evaluate this, we examined the ratio of T2 to T1 titers (T2/T1). For the purposes of analysis, T1 antibody titers that were 0 were assigned a value of 1 when used to determine the T2/T1 ratio.

As listed in Table 4, a higher T2/T1 ratio was significantly associated with moderately or poorly differentiated tumors as compared with tumors with a well-differentiated histology (244 ± 757 *v* 24 ± 64, $P = .011$), which is consistent with what was noted with T2 titers. Overall, patients with a high T2/T1 ratio were less likely to recur ($P = .010$). This association was also noted to a lesser degree when patients were subgrouped by treatment. The failure to achieve statistical significance in all subgroups may be due, in part, to the smaller number of patients analyzed. One additional confounding variable was that in the parent clinical trial[17] from which these serum samples were obtained, patients who did not respond to chemotherapy were immediately crossed over onto the surgical arm. Those patients fared better than individuals who had an incomplete response to chemotherapy and subsequently recurred.

Association Between Antibody Titers, Response to Chemotherapy, and Survival

Antibody titers were evaluated for their relationship to chemotherapy (Table 5). No statistically significant associations were noted with T1 or T2 titers. However, patients with a low T2/T1 ratio were found to have a higher

proportion of compete and partial responses ($P = .037$). Antibody titers were also evaluated for their relationship to overall and disease-free survival (Table 6). No statistically significant associations were observed in T1 or T2 antibody titers. Despite the association between the T2/T1 ratio and the risk of recurrence and response to chemotherapy, no statistically significant correlations with survival were observed. As noted earlier, this may be due, in part, to the fact that patients who did not respond to chemotherapy were crossed over onto the surgical arm. The only exception to this was seen in patients treated with surgery. In that group, no patients with a high T2/T1 ratio have recurred (follow-up duration, 13 to 65 months). However, only seven patients are in that group.

Table 6. Proportion of Patients Disease-Free at 2 Years by Titer*

Treatment Group and Stage	Antibody Titer ≤ Mean	Antibody Titer > Mean	P
Chemotherapy patients			
T1	.60 ± .08	.50 ± .13	.62
T2	.64 ± .09	.51 ± .11	.96
T2/T1	.59 ± .07	.58 ± .19	.61
Surgical patients			
T1	.79 ± .08	.69 ± .11	.17
T2	.73 ± .09	.79 ± .09	.66
T2/T1	.72 ± .07	1.00 ± 0	*
All patients			
T1	.70 ± .06	.60 ± .09	.28
T2	.69 ± .06	.64 ± .07	.79
T2/T1	.65 ± .05	.86 ± .09	.16

NOTE. Values are proportion of patients disease-free ± SE of the estimate.

*No patients with T2/T1 titers above the mean have died.

DISCUSSION

A major goal of this study was to determine if levels of circulating immune complexes and host-derived antibody were associated with prognosis in patients with SCCHN. The parent trial from which the serum specimens were obtained presented a unique opportunity to study a well-defined, homogeneous population of patients with SCCHN. A secondary aim of this study was to confirm the previous association between circulating immune complex levels and response to chemotherapy. As noted, patients whose C1q levels were greater than 115 μg/mL (mean ± 2 SD of normal C1q levels) were less likely to respond to chemotherapy, confirming the observations we made previously.[11] These results are also consistent with a report by Carpenter et al,[26] who noted, in acute myelogenous leukemia, that elevated C1q levels were associated with a decreased response to induction chemotherapy.

The antibody titer results reported here differ significantly from our previous serologic study in melanoma.[27] In that study, 43 stage I and II patients who were clinically free of disease were evaluated for the presence of antibody directed against melanoma in either native or AD&U serum. It was found that elevated antibody titers were associated with eventual relapse ($P = .0001$). In the present study, not only were patients with a very different histology studied, but all had their tumors present at the time serum samples were obtained. We have never evaluated antibody reactivity in patients with gross disease. While our previous serial serologic studies in both melanoma and SCCHN[14,16] were able to predict the development of recurrence, the limited number of patients precluded a more extensive evaluation.

In this current study, no significant correlations between T1 or T2 titers and clinical outcome were noted. The only exception to this was the relationship between T2 titers and the degree of differentiation. Higher T2 titers were associated with moderately or poorly differentiated tumors ($P = .0001$). This would suggest that more undifferentiated tumors elicit a stronger humoral response in the host or possess a greater quantity of the SCCHN-associated antigen. It also implies that the antigen detected by patients with SCCHN is less likely to be a normal cell constituent, which is consistent with the specificity analysis we have performed on seven autologous systems.[3,16]

We noted that there was marked variation in the relative increase of T2 titers following AD&U. In previous studies, we have shown that AD&U will remove free and immune complex–bound antigen without significantly affecting antibody reactivity.[13] A marked increase in T2 titers rel-

Table 5. Association Between Antibody Titers and Response to Chemotherapy

	T1					T2					T2/T1				
	≤ Mean		> Mean			≤ Mean		> Mean			≤ Mean		> Mean		
Response	No.	%	No.	%	P	No.	%	No.	%	P	No.	%	No.	%	P
CR	30	12	31	5		29	12	21	5		34	16	12	1	
PR	53	21	44	7		45	14	54	13		51	24	38	3	
NR	17	7	25	4	.865	16	5	25	6	.250	15	7	50	4	.037
Mean titer	332 ± 419					1,824 ± 1,695					243 ± 771				

ative to T1 titers would reflect a greater degree of antibody bound to circulating antigen. The T2/T1 ratio is, therefore, distinct from the measurement of T2 alone and is a more accurate reflection of the presence of tumor-associated immune complexes. Overall, patients with a high T2/T1 ratio were less likely to recur ($P = .010$). This association was also noted to a lesser degree when patients were subgrouped by treatment. The association between patients whose T2/T1 ratios fell above and below the mean and disease-free survival was also analyzed. Due to the small number of patients available for analysis, statistically significant associations were not as evident. However, in the patients randomized to the surgery arm, no patient whose T2/T1 ratio was greater than the mean has recurred (follow-up duration, 13 to 65 months). Because no events have occurred in the high T2/T1 group, a P value cannot be assigned.

The association noted between a low T2/T1 ratio and a higher response to induction chemotherapy was somewhat surprising given the associations we have noted with a high T2/T1 ratio and risk of recurrence. This apparent contradiction can be ascribed to the design of the parent clinical trial.[17] In that trial, patients who did not respond to chemotherapy were immediately crossed over onto the surgical arm. Those patients fared better than individuals who had an incomplete response to chemotherapy and subsequently recurred. This would account for the conflicting results reported here and is compatible with the outcome noted in the parent clinical trial. If the T2/T1 ratio is indicative of the level of tumor-associated immune complexes, then these results are consistent with our observations of C1q levels and response to chemotherapy both here and in the previous report by Schantz et al[11] noting that increased levels of immune complexes are associated with less of a response to induction chemotherapy.

Before drawing conclusions about the role of circulating immune complexes and tumor-associated antigens, questions regarding amount of antigen shed by individual tumors and the avidity and specificity of antibody binding need to be addressed. While it would be preferable to measure directly the amount of free and bound antigen present in the patient's serum, a monoclonal antibody capable of detecting the SCCHN-associated antigen is not available. The limited quantity of serum available, combined with the difficulties in standardization, precludes its use as a direct measure of circulating antigen. Although we have purified a SCCHN-associated antigen,[19] the quantity available at present is too limited to permit an evaluation of this scale. Finally, it is possible that a different antigen may be detected by individual patients. These results provide additional impetus to ongoing studies, in our laboratory, to develop monoclonal antibodies that detect this antigen and other tumor-associated antigens detected by patients with SCCHN.

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